

Malignant transformation of immortalized transgenic hepatocytes after transfection with hepatitis B virus DNA

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Persistent infection by hepatitis B virus (HBV) is epidemiologically correlated with the prevalence of hepatocellular carcinoma, but its role in tumor development is not yet understood. To study the putative oncogenic potential of HBV, a non-malignant immortal mouse hepatocyte line FMH202 harboring metallothionein promoter-driven simian virus 40 large tumor antigen was transfected with HBV DNA. All stably transfected clones which replicated HBV displayed malignant growth characteristics in soft agar and were tumorigenic upon inoculation in nude mice. The nude mice tumors were histologically classified as differentiated or anaplastic hepatocellular carcinomas. As with human liver carcinomas, rearrangements of *in vitro* integrated HBV sequences were observed in the nude mouse tumors, and in tumor-derived cell lines. In one case, expression of viral core and surface antigens was blocked in the tumors, correlating with hypermethylation of the HBV genome. However, the expression of X gene was maintained in most tumors and tumor-derived cell lines. X protein was detected in nuclei by immune fluorescence and by immune blot. These results provide the first demonstration that HBV displays oncogenic potential in an experimental system. This system could be useful to functionally identify HBV genes which convey a tumorigenic phenotype.

Key words: hepatitis B virus/hepatitis B X protein/hepatocellular carcinoma/hepatocyte line/malignant transformation

Introduction

Chronic carriers of hepatitis B virus (HBV) experience a much higher risk of developing hepatocellular carcinoma (HCC) than individuals who are immune against HBV or not infected at all (Beasley and Hwang, 1984). Although the role of HBV in the etiology of HCC is not well understood, HBV appears not to be an acutely transforming virus because, in general, HCCs develop many decades after infection. Frequently HBV-associated HCCs contain one or several inserts of HBV DNA in their genome (Dejean *et al.*, 1986; Shafritz *et al.*, 1988). Occasionally, cellular growth

controlling genes are disturbed by these inserts (Möröy *et al.*, 1986), and activation of cell growth as a consequence of HBV insertions has been suggested to be a common underlying cause of HBV-associated malignancies. Rearranged viral and host DNA sequences have frequently been observed during late phases of HCC development (Nagaya *et al.*, 1987). Therefore, studies of fully developed tumors do not provide reliable information about early events occurring after HBV DNA integration into the cellular genome, which could be causally related to the formation of HCC. It would be important to study the influence of the HBV genome on cellular growth behavior early after integration into a suitable cell.

So far, only completely transformed cells have been successfully used for HBV transfection experiments to investigate HBV replication and gene expression (Sureau *et al.*, 1986; Sells *et al.*, 1987; Tsurimoto *et al.*, 1987). Transforming activities of HBV cannot be studied in such cells. However, primary hepatocytes in culture are not suitable for such studies owing to their short life span. Moreover, they rapidly lose most of their cell-type specific functions after cultivation (Jefferson *et al.*, 1984) and display low transfection efficiency (Höhne *et al.*, 1988). Recently, non-tumorigenic hepatocyte lines have been established from livers of transgenic fetal mice (Paul *et al.*, 1988). These mice harbored as a transgene the DNA construct SVΔeMGH which includes large tumor antigen (TAg) encoding sequences of simian virus 40 (SV40) under the transcriptional control of the liver specific mouse metallothionein I promoter (Palmiter *et al.*, 1985). From one of the fetal mouse hepatocyte lines, FMH202, a clone was derived which expressed TAg, but did not grow in soft agar and was not tumorigenic in nude mice (Paul *et al.*, 1988 and references therein). These cells are highly differentiated and express many liver-specific genes, and can be growth arrested in subconfluent cultures using a culture medium free of mitogens (Paul *et al.*, 1988). We co-transfected this immortalized cell line with dimeric HBV DNA and a selectable marker, established several clones which stably replicated HBV DNA and tested the oncogenicity of these clones by growth in soft agar and in nude mice. The nude mouse tumors which resulted from the HBV expressing hepatocyte clones showed many of the peculiarities which have been observed in HBV associated human HCC.

Results

Transfection of hepatocytes by HBV DNA

For transfection studies, plasmids pKSVHBV1 (Figure 1) and pKSVneo were constructed. The shuttle vector pKSV10 contains as bacterial plasmid sequence pBR322 and as eukaryotic vector parts of the early gene region of SV40 without the functional gene. A head-to-tail dimer of cloned HBV DNA, isolate 991, was inserted downstream of the SV40 enhancer and early promoter (Figure 1C). The HBV

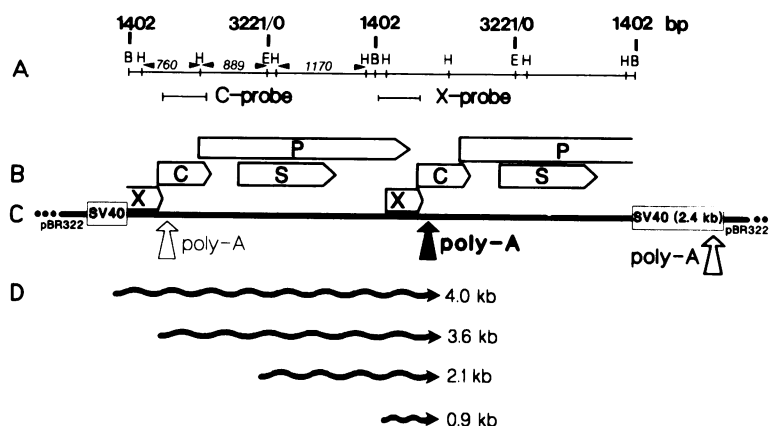


Fig. 1. Map of HBV expressing plasmid pKSVHBV1 and its transcripts. (A) Partial restriction map. The numbers on top refer to the *EcoRI* site (3221/0) and to the unique *Bam*HI site (1402) in HBV isolate 991. The numbers between the arrowheads show the sizes of expected fragments in base pairs (bp) after combined digestion with *Bam*HI (B), *Eco*RI (E) and *Hpa*II or *Msp*I (H). The map positions of subgenomic C and X probes for Northern and Southern blotting are also shown. (B) Position of the ORFs encoding core (C), polymerase (P), surface (S) and X protein (X). (C) Position of the dimeric HBV DNA within the shuttle vector pKSV10. (D) Mapping of RNAs expressed by HBV transfected mouse hepatocyte clones (for explanation see text). The positions of the three transcription termination sites are shown as vertical arrows (poly A).

insert starts slightly downstream of the first start codon in the X open reading frame (ORF). Figure 1B shows the position of all four HBV ORFs in pKSVHBV1. Plasmid pKSVneo contained the same vector sequence but the HBV DNA was replaced by a neomycin resistance gene. In previous studies, mouse fibroblast clones which had been stably co-transfected with the two plasmids were able to express those HBV proteins which are necessary for synthesis of HBV DNA by reverse transcription, and to secrete HBV DNA containing particles and viral antigens (Seifer, 1987).

Co-transfection of 4×10^6 FMH202-1 cells using the calcium phosphate co-precipitation method yielded 182 clones which were resistant to the neomycin analog G418. Thus, transfection efficiency was close to 10^{-4} . Of the 182 clones, 19 secreted the HBV surface antigen (HBsAg), 19 secreted soluble HBV core protein which is known as HBeAg and 17 clones secreted both HBV antigens. Cells which secrete HBsAg and HBeAg are likely to produce HBV particles also. Six of the 17 clones were brought into large-scale culture. Their supernatants were centrifuged to enrich HBV particles. The pellets from all six clones contained 8–25 pg/ml HBV DNA (see results of four clones in Table I). To prove the viral origin of the HBV DNA, aliquots were extracted with phenol before and after proteinase K digestion. Due to its mode of replication (Seeger *et al.*, 1986) the virion derived HBV DNA contains covalently bound protein which causes extraction into the phenol phase (Gerlich and Robinson, 1980). The major part of HBV DNA which was secreted by the six clones was indeed extractable by phenol unless it was digested with proteinase K (data not shown).

Malignant growth of HBV expressing clones

The FMH202 cell line and its clonal derivative FMH202-1 have maintained the functions of highly differentiated hepatocytes for at least 3 years. The cells multiply in medium free of serum in the presence of insulin, and can be growth arrested in insulin-free medium. After growth arrest the cells re-enter the cell cycle in response to insulin and display transient expression of proto-oncogenes *c-fos*, *c-myc* and *c-H-ras* prior to entering S phase (A.Höhne *et al.*,

Table I. Malignancy of HBV transfected hepatocyte clones

Clone transfected with	HBV DNA secreted (pg/ml)	Growth in agar ^a (%)	Tumors in nude mice ^b
Nothing	negative	negative	0/8
pKSV10 and pKSVneo 12 clones	negative	negative	not tested
pKSVHBV1 and pKSVneo			
H79	8 ± 2	72 ± 5	3/3
H109	12 ± 3	65 ± 8	2/3 ^c
H112	22 ± 5	48 ± 4	3/3
H119	25 ± 4	80 ± 5	3/3

^aPercentage of cells which grew to colonies >0.2 mm in 0.3% agar within 14 days (Höhne *et al.*, 1988).

^bFour-week-old NMRI *nu/nu* mice were inoculated subcutaneously with 10^7 cells in the back and observed for 30 weeks.

^cSlowly growing tumors which were first detectable after 20 weeks. Animals were handled in accordance with the German animal protection guideline.

unpublished results). The cells show an immortalized, non-malignant phenotype in culture (Paul *et al.*, 1988).

Four HBV expressing clones (H79, H109, H112 and H119) were analyzed if they showed altered growth behavior. In contrast to the parental cell line (Figure 2A), HBV-transfected clones multiplied independent of growth factors, and grew three times faster (doubling time 20 h) showing massive multilayer growth in tissue culture (Figure 2B). The clones formed large colonies in soft agar with high efficiency (Table I).

Transfection of cells by large amounts of vector DNA and selection of G418 resistant clones could theoretically induce malignant growth properties without HBV DNA. To test this possibility FMH202-1 cells were co-transfected exactly the same way as before except that the vector pKSV10 was used instead of pKSVHBV1. Transfection efficiency was similar to that above. Twelve neomycin resistant clones without HBV were explanted in soft agar but in contrast to the HBV-transfected clones they did not grow.

After demonstration of HBV dependent malignant growth *in vitro*, the four clones were injected into sets of three nude

mice. Three of the four clones consistently led to the formation of highly infiltrating and metastatic tumors (diameter >2 cm) within 8–10 weeks (Table I). Clone H109 appeared to be less tumorigenic and induced slowly growing tumors in two of three animals after 20 weeks. No tumor was detected after inoculation of the parental cell line into eight mice within 30 weeks. All nude mouse tumors were identified histologically as typical HCCs. In tumors derived from clone H119 a trabecular pattern with sinusoid-like spaces was found (Figure 2C), which is typical for differentiated HCCs. In other cases, infiltrating, less differentiated carcinomas were found, which contained pleomorphic cells with many atypical mitotic figures (Figure 2D). Cytoskeleton filaments were stained with monoclonal antibodies directed against cytokeratins 8 and 18, but not with cytokeratin 19, desmin and vimentin. This staining pattern is consistent with the hepatocellular origin of the tumors.

Integration, rearrangement and amplification of HBV sequences

The HBV genome was shown to be integrated in the genome of the four clones. Southern blots of undigested DNA from clones H112 (Figure 3A, lanes 1 and 4) and H119 (Figure 3B, lane 1) did not show detectable amounts of free viral or episomal HBV DNA, in spite of the secretion of HBV DNA containing particles. Southern blot is probably not sensitive enough to detect free viral DNA within the cells, because HBV DNA could only be well detected in the supernatants of the clones after 100-fold enrichment. The negative result for the cell extracts shows that there was no significant storage or intracellular amplification of free HBV DNA in the hepatocyte clones.

At passage 3, clone H112 contained at least three inserts of HBV DNA. This was shown by the number of fragments generated by digestion of genomic DNA with *Hind*III (Figure 3A, lane 3) which does not cleave HBV991 DNA (Böttcher *et al.*, 1982; Seifer, 1987). The generation of a 3.2 kb *Eco*RI fragment indicated that at least one complete copy of the HBV genome was present (Figure 3A, lane 2). This pattern of integration was stable *in vitro* because at passage 20 of clone H112 the same restriction fragments were found (Figure 3A, lanes 4–6). The nude mouse tumor H112-1, derived from clone H112, produced essentially the same pattern in the Southern blot (Figure 3A, lanes 7–9), but the larger fragments were not well defined due to unavoidable degradation of DNA in necrotic tumor cells. The cell line, derived from tumor H112-1, produced much stronger bands in the Southern blot (Figure 3A, lanes 11 and 12), which suggests amplification of the integrated HBV DNA. Moreover, a rearrangement had occurred, because the 12 kb *Hind*III fragment was not amplified in contrast to the 16 and 4.8 kb fragments (see arrows in Figure 3A).

In clone H119, two HBV inserts were identified as *Hind*III fragments of 18 and 12 kb (Figure 3B, lane 3). Again, a 3.2 kb *Eco*RI fragment (Figure 3B, lane 2) suggested the presence of at least one complete HBV genome. The three nude mouse tumors H119-1 to H119-3, which were induced by clone H119, showed a completely changed insertion pattern for HBV DNA (Figure 3B, lanes 4–6). In these tumors HBV DNA was found in three major *Hind*III fragments of 6, 5 and 4.6 kb. The presence of the larger *Hind*III fragment of clone H119 could not be established,

because the tumor DNA was partially degraded *in vivo*. However, in a clonal cell line, derived from trabecular tumor H119-1, the original fragments of 18 and 12 kb had disappeared and one very large fragment was found in addition to the three fragments which had already been detected in the tumors. The extensive rearrangement of the HBV DNA inserts during nude mouse passage becomes even more evident by the *Eco*RI cleavage pattern. It contained six major bands in the tumor derived cell line (Figure 3B, lane 9) which were not found in the original clone H119 (Figure 3B, lane 2).

In all cases, the integration pattern in tumors and tumor-derived cell lines of pKSVneo sequences (which had originally been co-transfected with the pKSVHBV1 construct) remained unchanged as compared with that in the transfected parental cell line (data not shown). Similarly, no changes in the integration patterns of the transgene SVΔeMG-202 in the cells or of *c-myc* were observed (data not shown).

HBV transcripts

Figure 4A, lane 1, shows the Northern blot of RNA from clone H112, and Figure 4B, lane 1, shows RNA from clone H119. Four sizes of HBV encoded RNA species were detected in the two clones: 4.0, 3.6, 2.1 and 0.9 kb. For the results of Figure 4, a genomic length HBV DNA probe was used. Further Northern blots of similar samples were done with core and X specific probes, the map position of which is shown in Figure 1A. The 3.6 kb RNA of clones H112 and H119 reacted both with the core and X probe (data not shown). This suggests that it maps as shown in Figure 1D, that it encodes HBe and/or core proteins and that it can serve as pregenome (Cattaneo *et al.*, 1984; Tiollais *et al.*, 1985).

The 2.1 kb RNA detectable in clones H112 and H119 did not react with the core probe but with the X probe. As shown in Figure 1D, it most likely corresponds to mRNA which expresses the small and middle sized HBs protein (Cattaneo *et al.*, 1983). The 0.9 kb RNA reacted only with the X probe. The largest observed RNA of 4 kb reacted not only with the core and X probe but also with a SV40 probe (data not shown). Thus, it was a chimeric RNA which was transcribed from the SV40 early promoter and terminated at the second polyadenylation signal of HBV, as suggested in Figure 1D. The data of Figure 4A and B (lanes 1) suggest that in cell culture the three HBV promoters were as active or even more active than the SV40 early promoter, and that only one of the three termination sites in construct pKSVHBV1 was active (black arrow in Figure 1C). The HBV RNA pattern was qualitatively identical in the other two clones except for H109 which had less X mRNA (data not shown).

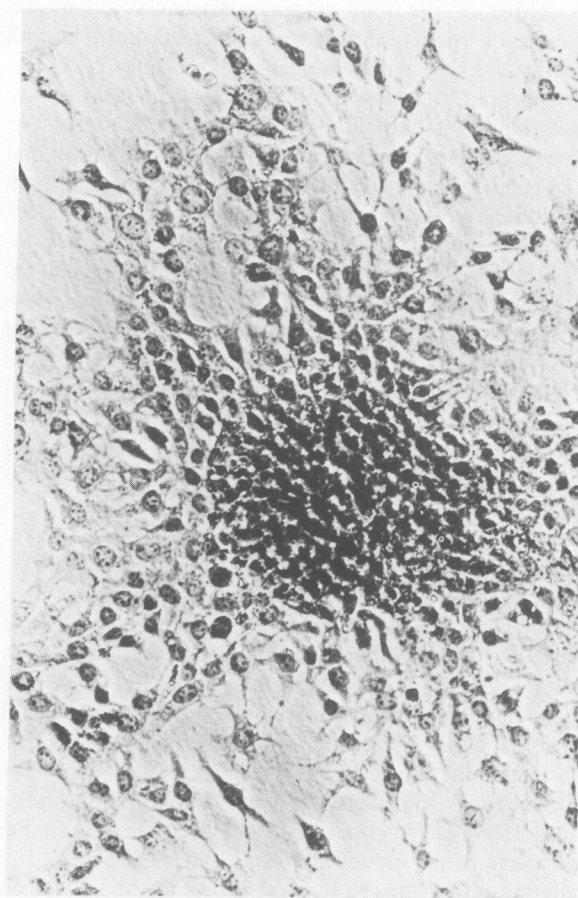
In the nude mouse tumors, HBV RNA patterns were highly variable. In tumor H112 (Figure 4A, lane 2) HBV RNA seemed to be absent. After hybridization with the X probe and long exposure of the autoradiograph two novel transcripts of ~7 and 8 kb became visible (Figure 4A, lane 4, arrows). After explantation of tumor H112-1 and cloning of a tumor derived cell line H112-1.1 the HBV RNAs were again expressed (Figure 4A, lane 3).

This observation suggested that a reversible mechanism suppressed the HBV expression of clone H112 *in vivo*. Modification of DNA by methylation at the 5' position of

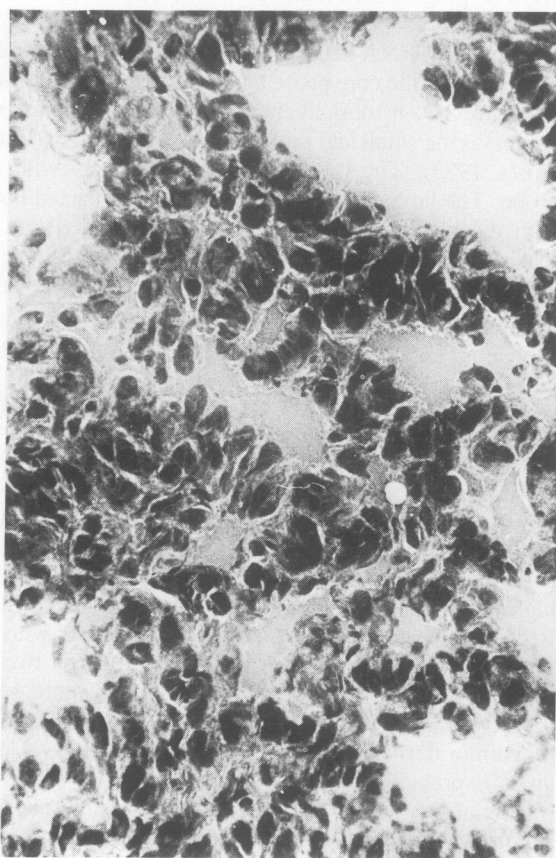
a



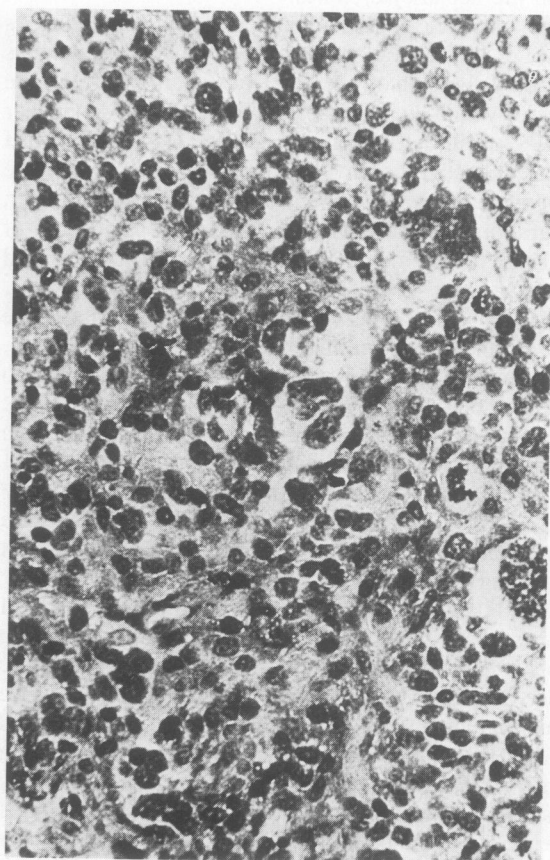
b



c



d



cytosine within CpG dinucleotides has been implicated as a mechanism frequently involved in modulating gene expression in many tissues including HCC (Miller and Robinson, 1983; Bowyer *et al.*, 1987). To study DNA methylation patterns, the DNA of clone H112, tumor H112 and the H112 tumor-derived cell line H112-1.1 was cleaved with *Bam*HI–*Eco*RI (Figure 5, lanes 1, 4 and 7) and subsequently digested with either *Hpa*II or *Msp*I. HBV991 DNA contains four *Hpa*II–*Msp*I cleavage sites (see Figure 1A, letters 'H') which would generate fragments of 760, 889 and 1170 bp from the 1819 and 1402 bp *Bam*HI–*Eco*RI fragments (see Figure 1A). As shown in Figure 5, *Msp*I indeed generated the three fragments in original clone H112 (lane 2), in the nude mouse tumor (lane 5) and in the tumor derived cell line (lane 8). Due to partial digestion by *Msp*I, the 1819, 1402 and a 1307 bp fragment were also visible. *Hpa*II which is inactive at methylated *Msp*I sites generated the 889 and 760 bp fragments in clone H112 (lane 3) and in the tumor derived cell line H112-1.1 (lane 9); however, in tumor H112 these fragments were completely absent (lane 6). The 1170 bp fragment was not detectable in all three DNA samples. Thus, it appears that the four *Msp*I–*Hpa*II sites became methylated in the nude mouse tumor and demethylated in the tumor derived cell line.

The three nude mouse tumors derived from clone H119 still expressed the 4.0 kb hybrid RNA and the 3.6 kb HBe/core RNA. The 0.9 kb X RNA was even more strongly expressed, but the 2.1 kb HBs RNA was virtually absent (Figure 4B, lanes 2–4). In the two cell lines H119-1.1 and H119-1.2 derived from tumor H119-1, the transcript patterns remained essentially unchanged (Figure 4B, lanes 5 and 6), but expression of HBs mRNA became weakly detectable in line H119-2.

HBV proteins in cell clones and tumors

The variable patterns of viral RNA in cells and tumors were reflected by the viral proteins detected in these materials. Due to the selection criteria it was clear that the small HBs protein and HBeAg were synthesized and secreted by the four clones. Moreover, the occurrence of secreted protein-linked HBV DNA implied that a functional HBV polymerase protein was generated. Using special enzyme immune assays, other structural proteins of HBV such as HBcAg, middle and large HBs protein were also found in the supernatants of transfected cell clones H79 and H119.

In the sera of all tumor-bearing nude mice no HBsAg, HBeAg or HBV DNA was detectable. This is in agreement with the absence of the 2.1 kb RNA in the tumors. Within the tumors there was also no HBsAg detectable. This excludes selective overexpression of large HBs protein which cannot be secreted (Marquardt *et al.*, 1987). No HBcAg or HBeAg was found in tumor H112 which also lacked the corresponding mRNA. In tumor H119 intracellular HBcAg was detectable by enzyme immune assay in agreement with the transcription data which showed a 3.6 kb RNA, but expression of the precore sequence which leads to secretion of HBeAg was apparently suppressed in the nude mice.

HBx antigen was searched for by immune fluorescence and immune blot. The available antibodies which were raised against partial peptides (Feitelson, 1986) did not generate clear staining patterns in our cells. The monoclonal antibody WC9-85 against HBx (M. Feitelson, submitted) also generated a weak non-specific staining in the cytoplasm of the parental cells and the HBV-transfected cells; however, in the transfected clone H119 an additional nuclear staining was visible (data not shown). Using the monoclonal antibody for immune blots, 17 and 14 kd HBx antigen bands were visible in the purified nuclei from HBV transfected clone H119 (Figure 6, lane 5) but not in the pKSVneo transfected control clone (lane 2). Non-specific staining of various larger proteins was seen in the cytosol (CS, lanes 3 and 6) and the cytoplasmic particulate fraction (CY, lanes 4 and 7) from both HBV containing and HBV free cells. Similar results on the presence of HBxAg were obtained with transfected mouse fibroblast clones (M. Seifer, in preparation).

Discussion

We report here that stable transfection of the non-malignant fetal mouse hepatocyte line FMH202 with HBV DNA results in the generation of lines which cause the formation of HCC upon inoculation in nude mice (Table I). The stable, immortalized growth properties of the parental FMH202 line are probably conferred by SV40-TAg, which is continuously expressed without leading to malignant growth (Paul *et al.*, 1988 and references therein). The transgenic mouse strain from which this hepatocyte line was derived is known to develop HCCs only at an age of ~3–4 months (Palmiter *et al.*, 1985). This suggests that SV40-TAg expression may not be sufficient for HCC induction and that presumably secondary events are required to induce the fully transformed hepatocyte phenotype, e.g. the accumulation of mutations, genomic rearrangements, complementation with activated cellular oncogenes, etc. (Gerber, 1986; Sandgren *et al.*, 1989). These secondary events have apparently not yet occurred in the FMH202 clone that was used for this study.

The results presented here indicate that in this system HBV displays oncogenic potential and suggest that the hepatocyte line FMH202 is suitable as an *in vitro* transformation system. It might therefore permit us to systematically dissect the HBV genome, and to functionally characterize gene(s) conveying oncogenic potential to hepatocytes. It is unlikely that SV40-TAg is responsible for the observed effects since cellular levels of SV40-TAg remained essentially unchanged in the transformed cells (data not shown). Furthermore, incubation of the parental cell line in medium with 10 μ M CdCl₂, which leads to a dramatic increase of TAg expression in the cells, did not induce the transformed phenotype (unpublished results). The FMH202 mouse hepatocyte line appears particularly suitable for studies involving HBV DNA, as specific hepatocyte functions are stably maintained (Paul *et al.*, 1988), thus reflecting many of the properties of the liver in the intact animal. This *in vitro* transformation system is in principle analogous to that

Fig. 2. Morphology of hepatocyte clones in culture (A, B) and histological sections of nude mouse tumors derived from them (C, D). (A) Non-malignant clone neo6 obtained after transfection with pKSVneo showing colonies of monolayer growth. (B) Clone H119 showing a focus of three-dimensional growth. (C) Nude mouse tumor H119 with sinusoid like spaces of trabecular HCC. (D) Nude mouse tumor H112, an undifferentiated solid nude mouse tumor showing multinucleated giant cells and abnormal mitotic figures. Photomicrographs of cultures (phase contrast, $\times 125$) (A, B) or of formalin fixed paraffin embedded sections ($\times 250$) were stained with hematoxylin/eosin (C, D).

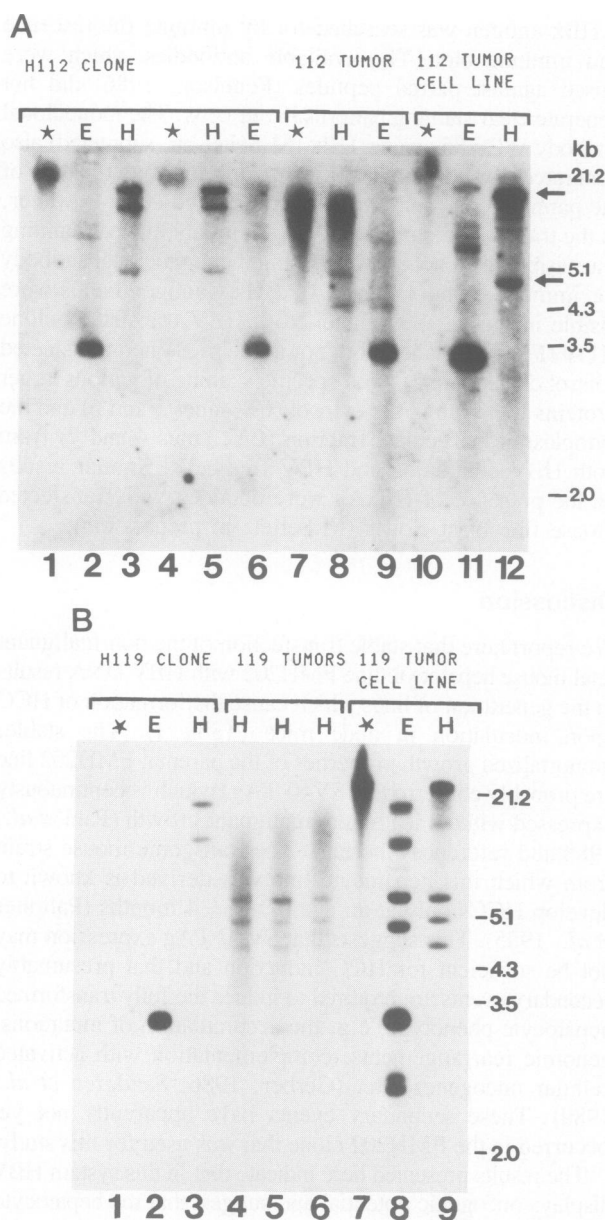


Fig. 3. Southern blot analysis of HBV DNA sequences in transfected clones, tumors and tumor-derived cell lines. Blots were hybridized to a full length pHBV991/M13 plus strand probe. Cellular DNA (20 μ g) was loaded on each lane without prior digestion (*), after digestion with *EcoRI* (E) or *HindIII* (H). Numbers at left show the migration of DNA fragments in kilobase pairs (kb). (A) Lanes 1–3: clone H112 after passage 3 *in vitro*; lanes 4–6: clone H112 after passage 20 *in vitro*; lanes 7–9: nude mouse tumor H112-1; lanes 10–12: cloned cell line H112-1.1 derived from nude mouse tumor H112-1. (B) Lanes 1–3: clone H119 after passage 3 *in vitro*; lanes 4–6: tumors H119-1, -2 and -3 all digested with *HindIII*; lanes 7–9: clonal cell line H119-1.1 derived from tumor H119-1.

involving NIH3T3 mouse fibroblasts, which led to the discovery of numerous cellular and viral oncogenes in focus assays in monolayer cultures.

In such assays the transformation frequency after transfection of appropriate oncogenes is variable, ranging between 10^{-4} and 10^{-5} . Although transformation frequencies were not accurately determined in the work described here, it is of particular interest that all four randomly chosen HBV-transfected clones showed tumorigenic potential, suggesting that HBV gene product(s) alone or in combination

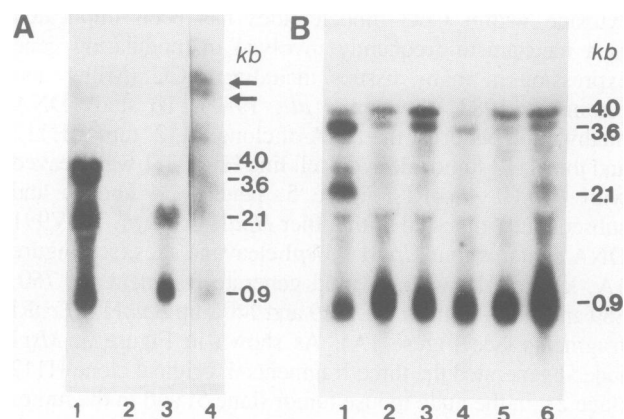


Fig. 4. Northern blot analysis of HBV-specific transcripts. Total cellular RNA (20 μ g) was loaded per lane. The blots were hybridized to a 32 P-labeled pHBV991 minus strand, or in one case to the labeled X probe mentioned in Figure 1A. (A) Lane 1: clone H112; lane 2: tumor H112-1; lane 3: cell line H112-1.1 derived from tumor H112-1; lane 4: tumor H112-1, hybridized with an X probe and exposed five times longer than in lane 2. The arrows show novel X transcripts. (B) Lane 1: clone H119; lane 2: tumor H119-1; lane 3: tumor H119-2; lane 4: tumor H119-3; lane 5: cell line H119-1.1 derived from tumor H119-1; lane 6: another cell line, H119-1.2, derived from tumor H119-1.

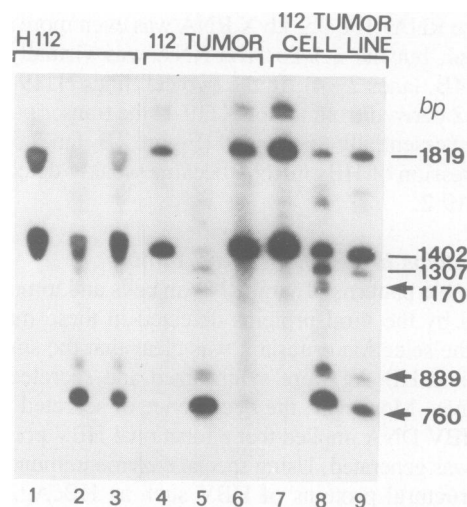


Fig. 5. Southern blot analysis of *HpaII* site methylation in clone H112 (lanes 1–3), tumor H112-1 derived from it (lanes 4–6) and the tumor cell line H112-1.1 derived from tumor H112-1 (lanes 7–9). Total cellular DNA was digested first with *BamHI* and *EcoRI*, thus generating fragments of 1819 and 1402 bp (lanes 1, 4 and 7). Thereafter DNAs were digested with *MspI* (lanes 2, 5 and 8) or with *HpaII* (lanes 3, 6 and 9). *MspI* would generate fragments of 1170, 889 and 760 bp, the map position of which is shown in Figure 1A. *HpaII* generated the same fragments, unless its sites are methylated as in tumor H112-1 (lane 6).

were sufficient for the transformation of the immortal FMH202-1 hepatocyte line. The transfection efficiency of our system for G418 resistance was 10^{-4} . Presuming that tumorigenicity would have been detected in all 17 HBV expressing clones, the transformation frequency would be 10^{-5} , which suggests that in this system HBV acts like a transfected oncogene. The absence of a detectable oncogenicity in the vector system suggests that the SV40 enhancer/promoter does not develop oncogenicity on its own. It remains to be tested which potentially oncogenic co-factors

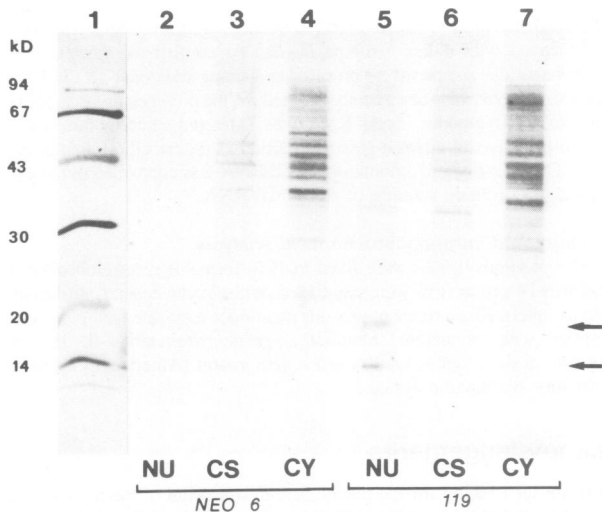


Fig. 6. Detection of X protein by immune blot using monoclonal antibody WC9-85. Nuclei (Nu), cytosol (CS) or cytoplasmic 100 000 g pellet (CY) were extracted from HBV-transfected clone H119 (lanes 5–7) or from control clone neo6 which had received only the selection marker (lanes 2–4). Lane 1: marker proteins. The arrows indicate the specifically stained X proteins in the nuclei of clone H119.

of FMH202-1 complement the oncogenicity of HBV to become manifest *in vitro* and *in vivo*. Studies currently performed suggest that other immortal cells without SV40-TAg can also be converted to higher malignancy by pKSVHBV1 (M.Seifer, unpublished).

The expression patterns of viral sequences in HBV-transfected tumors and in tumor-derived cell lines were highly variable. For example, viral gene expression in anaplastic, undifferentiated tumor H112 was nearly completely extinguished, whereas it was only partially blocked in the more differentiated trabecular tumor H119. These results are reminiscent of findings in human HBV-associated malignancies in the liver, where HBsAg expression occurs predominantly in liver tissue adjacent to the tumor, liver adenomas or in hyperplastic nodules, but not in highly dysplastic HCCs (Gerber, 1986). Presumably, liver-specific factor(s) acting in *trans* are required for the expression of certain viral genes, as shown by transient expression studies involving regulatory HBV sequences (Shaul and Ben-Levy, 1987; Karpen *et al.*, 1988; Chang *et al.*, 1989). Such factors may frequently get lost in the process of de-differentiation during HCC development, thus causing a reduction or a failure of HBV gene expression in the tumor. Therefore, studies on the expression of HBV sequences in developed HCCs will presumably not permit a rigorous analysis of the primary events responsible for hepatocyte transformation, which ultimately leads to HCC formation.

An interesting difference between the HBV-transfected clones used in this study and naturally infected livers are the high levels of X mRNA found to be present in the former. This mRNA has not been detected in livers of subjects acutely or persistently infected with human HBV (Will *et al.*, 1987). Very little X mRNA was found in woodchucks persistently infected with woodchuck HBV (Kaneko and Miller, 1988). X protein is known to activate transcription by acting on the HBV enhancer, but it also activates other viral and even certain cellular enhancers (Koike *et al.*, 1987; Spandau and Lee, 1988). However, it does not activate the

metallothionein promoter/enhancer (Zahm *et al.*, 1988) and it even inhibits the activity of SV40-TAg (Zahm *et al.*, 1988). Many viral *trans*-acting transcriptional activators, including adenovirus E1A, HTLV-1 pX or papillomavirus E6 and E7, are known viral oncogenes. In this respect it is remarkable that the highly tumorigenic clones H79, H112 and H119 contained more X mRNA than the less tumorigenic clone H109. However, it remains to be shown whether the expression of the X gene alone confers tumorigenic potential to FMH202 cells. Irrespectively, the X protein may also play an important role in the development of natural liver carcinomas. In HCCs the integrated HBV sequences are frequently linked with the cellular DNA at the 3' end of X gene or at sites upstream of the X gene (Nagaya *et al.*, 1987). Occasionally, this generates chimeric transcripts which have been shown to *trans* activate (Wollersheim *et al.*, 1988). In tumor H112-1 we have observed chimeric transcripts of 7 and 8 kb.

Recently, a second *trans*-activating protein has been identified in certain HBV integrates of human HCCs. This consists of truncated large or middle HBs protein (Kekulé *et al.*, 1990). The identification of the parts in the HBV genome which are oncogenic for FMH202 cells is currently under investigation. Results obtained so far are consistent with the hypothesis that the X protein is one factor which, however, is not sufficient.

The suppression in nude mouse tumor H112-1 of viral core gene expression as a consequence of DNA methylation is reminiscent of events occurring in natural tumors. Viral core proteins are known inhibitors of transcriptional activation (Twu and Schloemer, 1987), and may therefore inhibit X gene expression (Twu *et al.*, 1988). This may explain why HCCs are virtually absent in viremic HBV carriers who usually express large amounts of core/e protein.

A typical feature of many human HBV-associated HCCs is the occurrence of DNA rearrangements and amplification of integrated viral sequences (Nagaya *et al.*, 1987; Hatada *et al.*, 1988). The results shown here suggest that the observed rearrangements of HBV sequences within the cellular genome, but not necessarily of other genes known to be involved in cellular growth control like TAg, *c-myc* (results not shown), are consequences of a continuous process of intense selection for unrestricted cell growth both *in vivo* and *in vitro*. It is interesting that rearrangements do not seem to occur frequently in HBV-transfected clones growing in culture, but become increasingly prominent during tumor growth *in vivo* and in tumor-derived cell lines growing in culture. Thus, these results shed light on the process of tumor development and progression: initially, HBV sequences, when combined with appropriate co-factors, lead to the acquisition of the malignant phenotype of the cells. Subsequently, rearrangements and amplifications of viral sequences occur, thus providing selective growth advantages during the late phase of tumor development, i.e. during tumor progression.

Materials and methods

Cell lines, plasmids and antibodies

Hepatocyte line FMH202 was originally derived from fetal livers (day 19) of transgenic mice (strain SVΔeMGH-202) (Paul *et al.*, 1988). At passage 62, clone FMH202-1 was isolated, and used for the experiments described in this study.

Viral DNA of HBV was isolated from the plasma (no. 991) of a chronic

asymptomatic virus carrier (HBsAg subtype adw), cloned at its *Bam*HI site with pBR322 (Böttcher *et al.*, 1982), and dimerized at its unique *Bam*HI site (base 1402 relative to the *Eco*RI site). The head-to-tail dimer was ligated into the *Bgl*II site of vector pKSV10 (Pharmacia), yielding pKSVHBV1 (Figure 1). A similar construct, pKSVneo, which confers resistance to the neomycin analog G418 (Gibco), was prepared by inserting the *Tn5* gene (derived from pNEO, Pharmacia, by cleavage with *Bam*HI and *Hind*III) into the *Bgl*II site of pKSV10 (Seifer, 1987).

A 611 bp *Nco*I–*Bgl*II fragment excised from pHBV991MB which had been recloned at its *Eco*RI site served as X probe, and a 791 bp *Hgi*AI–*Hae*III fragment excised from pHBc20 (Uy *et al.*, 1986) was used as core specific probe (see Figure 1A). Single stranded plus (+) or minus (–) strands of pHBV991, cloned in M13 vectors were used as strand specific probes.

To identify X protein in cell extracts, immune fluorescence and immune blot analyses were performed using a monoclonal anti-HBx (WC9-85) antibody (M.Feitelson, submitted).

DNA transfection and selection of clones

Cell line FMH202-1 was cultured in serum-free chemically defined MX83 medium (Hoffmann *et al.*, 1989) with 40 μ M arginine in monolayer cultures in the presence of 10 μ g/ml insulin (Sigma) and 300 ng/ml hydrocortisone (Jefferson *et al.*, 1984). Subconfluent cultures (5×10^5 cells per 6 cm dish) were transfected with 10 μ g pKSVHBV1 and 1 μ g pKSVneo by using the calcium phosphate/DNA co-precipitation procedure followed by glycerol/butyrate treatment to increase transfection efficiency (Gorman, 1985) and selected for growth in MX83 medium containing 250 μ g G418/ml (Gibco). Resistant colonies were transferred to microtiter plates and supernatants were tested after growth to confluency for HBsAg and HBcAg using sandwich enzyme immune assays (Enzygnost, Behringwerke). Six HBsAg and HBcAg secreting colonies were further cloned by limited dilution.

For detection of secreted HBV DNA, supernatants of these cells were pelleted at 100 000 g overnight, resuspended in 1% of the original volume TNE, and assayed by dot blot as described by Zyzik *et al.* (1986). To distinguish pelleted nuclear HBV DNA sequences from virus particle associated DNA, the assay was done after phenol extraction of samples with and without digestion by proteinase K (Gerlich and Robinson, 1980). Pre-antigen and HBcAg were detected in preparations by sandwich enzyme immune assays (Marquardt *et al.*, 1987).

Nude (*nu/nu*) NMRI mice (Zentralinstitut, Hannover, FRG) were inoculated subcutaneously with cells in the back and inspected weekly. Tumors were explanted, minced, treated with collagenase (Höhne *et al.*, 1988; Hoffmann *et al.*, 1989), placed in culture as single cell suspensions in MX83 medium in the presence of fetal calf serum (FCS), cloned and passaged twice weekly.

Subcellular fractionation and immune blot analysis

Cells (1×10^7) were homogenized in 4 ml of 0.25 M sucrose/TKM (50 mM Tris–HCl, pH 7.5, 25 mM KCl, 5 mM MgCl₂, 1 mM PMSF, 10 μ M leupeptin hemisulfate) by 24 strokes in a Dounce homogenizer on ice. Nuclei were sedimented for 10 min at 1000 g, resuspended in 7 ml 56% (w/w final concentration) sucrose/TKM, layered on 5 ml 60% (w/w) sucrose/TKM and centrifuged in a swinging bucket rotor (SW40) at 100 000 g (1 h; 5°C). The resulting pellets were resuspended in TNE/0.1% Tween 20. Supernatants of the 1000 g homogenate were layered on a cushion of 20% (w/w) sucrose/TKM and centrifuged at 100 000 g (1 h; 5°C). This pellet was considered as particulate cytoplasmic fraction (CY in Figure 6), the supernatants as cytosol (CS in Figure 6). Samples of 25 μ g protein were subjected to SDS–PAGE after denaturation by boiling in Laemmli sample buffer as described previously (Gültekin and Heermann, 1988). The separated proteins were transferred electrophoretically to GVHP membrane (Millipore). After saturation with 20% FCS the membranes were incubated for 2 h with WC9-85 monoclonal anti-HBx antibody containing hybridoma supernatant. After five washes (0.5% Tween 20 in PBS; each 10 min), the membranes were incubated with peroxidase labeled anti-mouse immunoglobulin (Dakopatts) in 20% FCS in PBS for 2 h, and subsequently stained with diaminobenzidine/H₂O₂.

Southern and Northern blot hybridization analysis

Nucleic acids were isolated from tissues or cells as previously described (Höhne *et al.*, 1988) by using a modification of the guanidinium isothiocyanate procedure (Chirgwin *et al.*, 1979). DNA samples were digested with appropriate restriction endonucleases (Boehringer, Mannheim), and analyzed by electrophoresis on agarose gels. After blotting, filters were hybridized with [³²P]dCTP-labeled DNA. DNA probes were labeled by nick translation as described (Rigby *et al.*, 1977) or by random

oligonucleotide priming (Feinberg and Vogelstein, 1983). To study the extent of DNA methylation of inserted HBV sequences, genomic DNA fragments were cleaved with either *Msp*I or *Hpa*II. To confirm completeness of digestion, parallel samples were prepared containing additional ϕ X174 DNA (1 μ g/ml), cleaved and subsequently checked for the presence of the expected phage DNA fragments. Total RNA was extracted, electrophoresed in denaturing glyoxal agarose gels and blotted as described (Höhne *et al.*, 1988). The integrity and amount of loaded RNA was controlled in the gels by ethidium bromide staining of ribosomal RNAs.

Histology and immunocytochemical analysis

Samples of tumor tissues were fixed in 10% formaldehyde, embedded in paraffin and 4 μ m sections were stained with hematoxylin/eosin. Cytoskeleton proteins, intermediate filament proteins and tumor-associated antigens were identified with monoclonal antibodies against cytokeratins 8, 18, 19, vimentin, desmin, actin, tubulin and α -fetoprotein (Amersham) by using the biotin–streptavidin system.

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